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End-Ischemic Machine Perfusion Reduces Bile Duct Injury In Donation After Circulatory Death Rat Donor Livers Independent of the Machine Perfusion Temperature

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Abstract

Background: A short period of oxygenated machine perfusion (MP) after static cold storage (SCS) may reduce biliary injury in donation after circulatory death (DCD) donor livers. However, the ideal perfusion temperature for protection of the bile ducts is unknown. In this study, the optimal perfusion temperature for protection of the bile ducts was assessed.

Methods: DCD rat livers were preserved by SCS for 6 hours. Thereafter, 1 hour of oxygenated MP was performed using either hypothermic (HMP), subnormothermic (SNP) or with controlled oxygenated rewarming (COR) conditions. Subsequently, graft and bile duct viability were assessed during 2 hours of normothermic *ex situ* reperfusion.

Results: In the MP study groups, lower levels of transaminases, LDH and TBARS were measured compared to SCS. In parallel, mitochondrial oxygen consumption and ATP production were significantly higher in the MP groups. Biomarkers of biliary function, including bile production, biliary bicarbonate concentration and pH, were significantly higher in the MP groups, whereas biomarkers of biliary epithelial injury (biliary gamma-GT and LDH) were significantly lower in MP preserved livers. Histological analysis revealed less injury of large bile duct epithelium in the MP groups, compared to SCS.

Conclusion: Compared to SCS, end-ischemic oxygenated MP of DCD livers provides better preservation of biliary epithelial function and morphology, independent of the temperature at which MP is performed. End-ischemic oxygenated MP could reduce biliary injury after DCD liver transplantation.

Introduction

Ischemic cholangiopathy, also known as non-anastomotic biliary strictures (NAS), is one of the most prevalent and troublesome complication after liver transplantation. During NAS formation, in particular, the large (extrahepatic) bile ducts become fibrotic and/or necrotic. Patients with NAS may suffer from recurrent jaundice and episodes of cholangitis and retransplantation may be the only curative treatment (1). The combination of ischemia and ischemia/reperfusion (I/R) injury has been shown to be a major risk factor for the development of NAS after transplantation (2). The combination of ischemia and I/R injury can lead to impaired regeneration of the biliary epithelium with subsequently NAS formation as clinical consequence (3,4).

A short period of end-ischemic machine perfusion (MP) after the regular period of static cold storage SCS has been shown to reduce I/R injury, compared to static cold storage (SCS) alone (5). Although the exact mechanisms underlying the protective effects of end-ischemic MP are not fully known, an important feature of end-ischemic MP is the resuscitation of mitochondrial respiration and resynthesis of cellular adenosine triphosphate (ATP) (5,6). Restoration of cellular ATP improves metabolic function after reperfusion, making the hepatocytes and cholangiocytes more resistant to the effects of I/R injury (5,6). Although recent data from animal models and (discarded) human donor livers have provided promising results suggesting that end-ischemic MP has relevant protective effects on the bile ducts of DCD liver grafts, the most optimal perfusion temperature during end-ischemic MP has not been investigated (7-17). Aim of this study is, therefore, to assess the optimal perfusion temperature during end-ischemic oxygenated MP for protection of the large bile ducts against I/R injury in a DCD rat liver model.

Materials and methods

Animals

Male Lewis rats (LEW/Han®Hsd) (290-320 g) were obtained from Harlan Laboratories (Boxmeer, the Netherlands). Animals received care according to the guidelines set by the US National Institutes of Health (1985). The study protocol was approved by the Institutional Animal Care and Use Committee of the University of Groningen, the Netherlands (IACUC-RuG).

Experimental Design

Thirty rat livers were divided into four experimental groups and a reference group (n = 6 per group). The four experimental groups were used to study the effects of end-ischemic MP, while the reference group was used for *in vivo* collection of bile during 2 hours of anesthesia (Figure 1). In the four experimental groups, livers were procured from DCD donors and subsequently preserved by SCS in histidine-

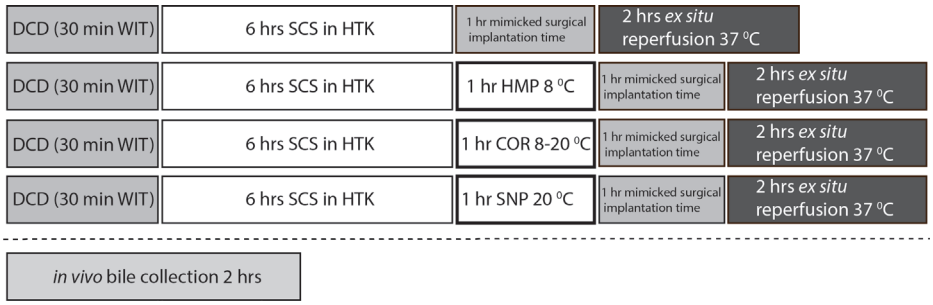


Figure 1: Schematic representation of the experimental groups to examine the effects of end-ischemic machine perfusion (MP) at three different perfusion temperatures: hypothermic 8°C (HMP), controlled oxygenated rewarming 8-20°C (COR), and subnormothermic 20°C (SNP). A group of DCD livers underwent only static cold storage without end-ischemic MP (SCS alone). For normal values of bile composition, bile was collected 2 hours *in vivo* in the reference group. All groups contained 6 rat livers.

tryptophan-ketoglutarate (HTK) preservation solution (Custodiol, Essential Pharmaceuticals, Ewing, NJ, USA) for 6 hours. To study the effects of end-ischemic MP, the 6 hours of SCS were followed by 1 hour end-ischemic oxygenated MP at 3 different perfusion temperatures. The fourth group consisted of livers that were preserved by 6 hours of SCS alone (Figure 1). MP was performed at hypothermic conditions (8°C [HMP]), subnormothermic conditions (20°C [SNP]) or with controlled oxygenated rewarming [COR], using Belzer machine perfusion solution (MaPerSol, Bridge-to-Life, Ltd. Northbrook, IL, USA). During COR, the temperature of the perfusate was kept at 8°C for the first 20 minutes and then gradually increased from 8 to 20°C in the next 20 minutes. For the last 20 minutes of the COR phase, the perfusion temperature was kept at 20°C. Prior to reperfusion, all livers were flushed with 10 mL of cold saline and subsequently stored on a petri dish, covered with a wet gauze at room temperature for 60 minutes, to mimic surgical implantation of the liver. Subsequently, liver grafts were reperfused *ex situ* for 2 hours with a perfusion fluid consisting of 25 mL human red blood cell concentrate (final hematocrit 25%) (Sanquin, Amsterdam, the Netherlands), 53.9 mL William's Medium E solution (Life Technologies Europe, Bleiswijk, the Netherlands), 20 mL human albumin (200 g/L Albuman, Sanquin, Amsterdam, the Netherlands), 1 mL insulin (100 IE/mL Actrapid, Novo Nordisk, Alphen aan den Rijn, the Netherlands) and 0.1 mL unfractionated heparin (5000 IE/mL, LEO Pharma A/S, Ballerup, Denmark), adding up to a total volume of 100 mL.

In vivo Bile Collection and Procurement of DCD Donor Livers

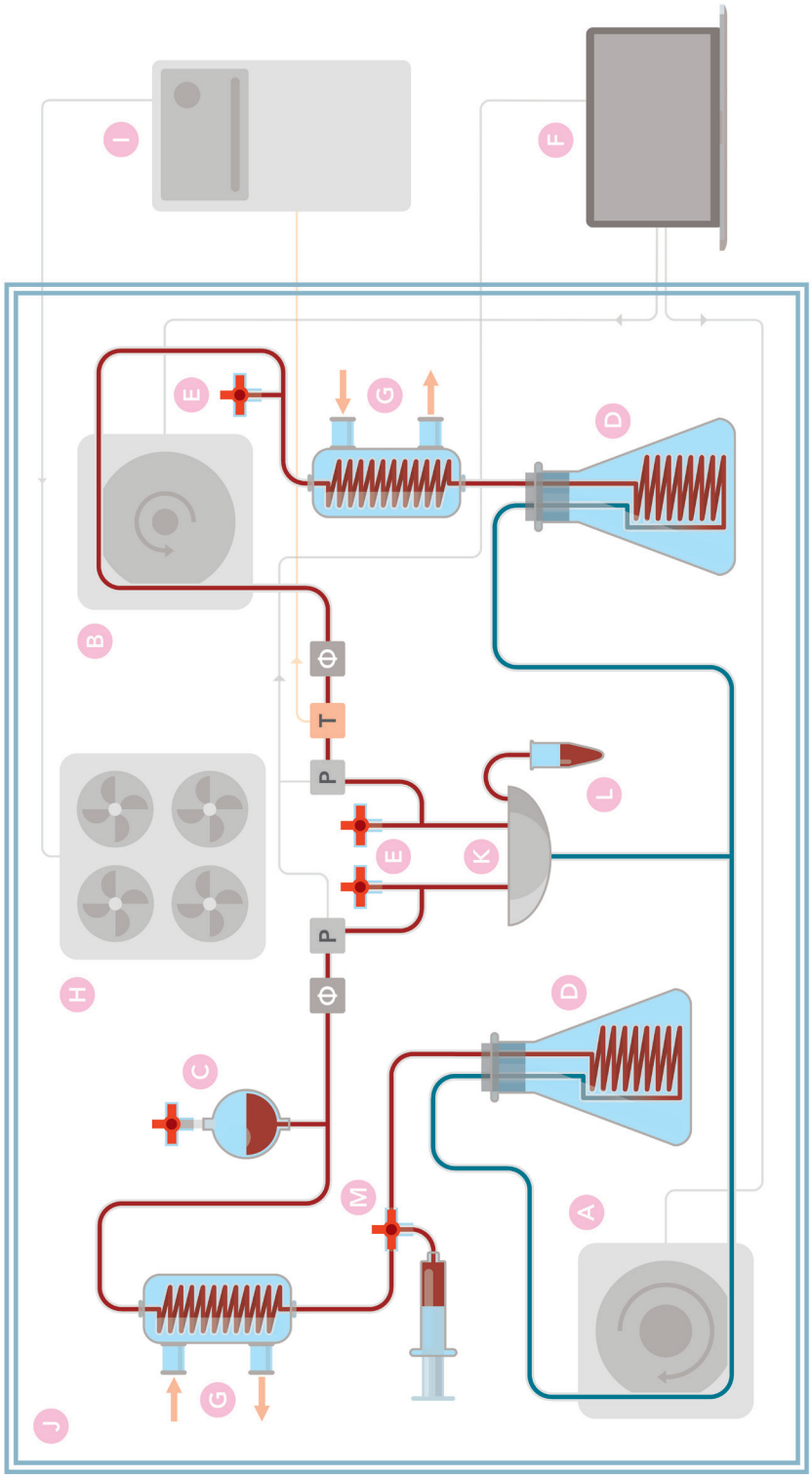
Inhalation anesthesia with isoflurane and oxygen was used before and during the procurement (2-3% isoflurane). First, the large bile duct was cannulated. For the reference groups, the rats were supported with mechanical ventilation and bile was collected for 2 hours in Eppendorf tubes. The procedures of DCD and *in situ* WIT (30 min) have been described previously (18). In brief, 1 mL 0.9% NaCl with 500 IU of heparin was administered via the dorsal penile vein. After heparinization,

cardiac arrest was induced by external compression of the heart (tamponade) until contractions ceased. Subsequently, the aorta was closed using a vascular clamp rostral from the heart for 30 minutes. The thoracotomy and laparotomy wounds were covered with gauze, moistened with 0.9% NaCl. To prevent cooling, a heating lamp was placed above the rat. After 30 minutes *in situ* WIT, the hepatectomy was performed by ligation of the splenic vein, mesenteric artery, and mesenteric vein. Thereafter, the celiac trunk was cannulated. After clamping of the infra-hepatic vena cava and the portal vein, the portal vein was cannulated and via the portal vein cannula, the liver was flushed *in situ* with 10 mL 0.9% NaCl (37°C). Subsequently, the supra-hepatic vena cava was transected, followed by a cold flush out with 5 mL HTK preservation solution (4°C) via the portal vein cannula. The liver was removed and flushed with an additional 20 mL of cold (4°C) HTK via the portal vein cannula and 5 mL of cold (4°C) HTK via the hepatic artery (celiac trunk cannula) before preservation by SCS.

Static Cold Storage, End-Ischemic Machine Perfusion and Ex situ Reperfusion

For SCS, livers were stored in bags with ice-cold HTK (4°C) on melting ice for 6 hours. End-ischemic MP and *ex situ* reperfusion of rat donor livers was performed with a liver machine perfusion system that enabled dual perfusion via both the hepatic artery and the portal vein using a closed circuit (Figure 2). Two roller pumps (Ismatec ISM404 + ISM719 and MS-2/6-160; IDEX Health & Science, Wertheim-Mondfeld, Germany) provided pulsatile flow through the hepatic artery and continuous flow through the portal vein. Continuous flow to the portal vein was achieved by the combination of elastic tubing and a pulse damper to remove pulses from the roller pump. Two tubular membrane oxygenators provided oxygenation of the perfusion solution and removal of CO_2. Perfusion box and perfusate temperature was maintained stable at the indicated temperatures using a thermostat pump (Huber, Offenburg, Germany) and radiator/ventilator combination (Freezing Hardware, Losser, the Netherlands). The system was pressure-controlled by a computer algorithm allowing autoregulation of blood flow through the liver, with constant pressure at variable flow rates. In-line sensors monitored flow, pressure, and temperature. Data were displayed in real-time on a connected laptop.

During HMP (8°C), the portal pressure was 3 mmHg and mean arterial pressure was 25 mmHg. During SNP (20°C), the portal pressure was set at 4 mmHg and mean arterial pressure at 40 mmHg. During the first 20 minutes of COR, the same portal and arterial pressure were used as during HMP. Thereafter, the perfusion solution was gradually warmed up to 20°C in 20 minutes and for the last 20 minutes the pressures were set at levels used during SNP (4 and 40 mmHg, respectively). *Ex situ* reperfusion (37°C) was performed with a mean arterial pressure of 110 mmHg and 11 mmHg at portal side. During end-ischemic MP and *ex situ* reperfusion, the perfusion fluid was oxygenated with 100% 02 and the pO2 was 60-80 kPa (450-600 mmHg), as described previously (8,11,15,19).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◀ **Figure 2: Schematic presentation of the rat liver machine perfusion system providing a combination of arterial and portal perfusion of the liver.** Two roller pumps provide a continuous flow to the portal vein (A) and a pulsatile flow to the hepatic artery (B). Pulses in the portal flow were eliminated with elastic tubing and a pulse damper (C). Two tubular membrane oxygenators provide oxygenation of the perfusion solution, as well as removal of CO_2 (D). Several bubble traps (three-way connectors) were used to eliminate air bubbles in the perfusion solution (E). Flow (Φ) and pressure (P) were detected by in-line sensors and data were displayed and analyzed in real-time on a connected laptop (F). The perfusion temperature was maintained constant by two heat exchangers (G) and a radiator/ventilator combination (H), all connected to the thermostat pump (I). For real time control of the perfusion temperature, one in-line temperature sensor (T) was connected to the thermostat pump. The isolated box encapsulated the perfusion system (J) preventing loss of warm or cold air. The rat liver was placed into an organ chamber (K). Bile was collected in Eppendorf tubes (L). By the three-way connector at the portal side, samples of the perfusion solution were taken every 30 min for analysis of the perfusate (M).

Biochemical Markers of Function and Injury

During *ex situ* reperfusion, flow, and temperature were registered every 10 minutes. Before reperfusion and after every 30 minutes of reperfusion, samples were taken from the perfusion fluid. Samples were centrifuged (2700 g for 5 min at 4°C) and the supernatant was collected, frozen and stored at -80°C for determination of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH), using standard biochemical methods.

Bile production was measured at 60-minute intervals by weighing Eppendorf tubes in which bile was collected from the biliary drain. Biliary epithelial cell function was assessed by measuring pH and bicarbonate concentration in bile (20). For this purpose, bile samples were collected under mineral oil and analyzed immediately using an ABL800 FLEX analyzer (Radiometer, Brønshøj, Denmark). Biliary concentration of gamma-glutamyl transferase (gamma-GT) and LDH were measured as biomarkers of biliary epithelial cell injury (21), and biliary bilirubin concentration was measured as biomarker of hepatocellular secretory function (21), using standard biochemical methods.

Thiobarbituric acid reactive substances (TBARS) were measured in perfusate samples after 2 hours of reperfusion, as a marker for oxidative stress. The method for TBARS measurements has been described previously (12).

Isolation of Mitochondria and Mitochondrial Oxygen Consumption Analysis

Mitochondria isolation and mitochondrial oxygen consumption analysis were performed after 2 hours *ex situ* reperfusion. The protocol for isolation and oxygen consumption measurement has been described before (22). In brief, oxygen consumption was measured with a Clark type electrode (Strathkelvin Instruments LTD, North Lanarkshire, UK). This electrode was placed in a double walled respiration chamber with continuous stirred suspension of the isolated mitochondria (2 mg/mL) and oxygen consumption buffer at 37°C. Basal oxygen consumption of the mitochondria (state 2 respiration) was measured after addition

of glutamate (10 mM) and malate (2 mM) as substrates to stimulate complex I, III, and IV. Maximal respiration rate (state 3 respiration) was measured after addition of adenosine diphosphate (ADP) (5 mM), which causes a sudden burst of oxygen uptake as the ADP is converted into ATP by ATPase synthase (complex V). Oligomycin (2.5 μM) was used to stop ATPase synthase by blocking complex V (defined as state 4 respiration). Rates of oxygen consumption were expressed as μmol O_2/min/mg liver. The mitochondria respiratory control ratio (RCR) was defined as the ratio of state 3 and state 4. The function of the RCR is to reflect the viability of the mitochondria (23).

Adenosine triphosphate (ATP) Extraction and Measurement

Hepatic concentration of ATP was used as an indicator of the energy status of grafts after 2 hours of *ex situ* reperfusion. Method for extraction and measurement has been described previously (12).

Histological Evaluation of the Large Bile ducts

After 2 hours of *ex situ* reperfusion, a segment of the large bile duct proximal from the tip of the biliary catheter (and therefore not mechanically injured) was dissected and stored in 10% formaldehyde for inclusion in paraffin. Paraffin-embedded slides were cut with 0.5 mm interspaces, resulting in 3 slides per bile duct. In addition, staining was performed with hematoxylin and eosin (H&E) staining. Large bile duct injury was semi-quantified using a systematic scoring system described by Hansen *et al.* (24) with adjustments according to op den Dries *et al.* (4). All bile duct sections were examined in a blinded fashion by two investigators (ACW and SLM) under supervision of an experienced liver pathologist (ASHG) using light microscopy.

Statistical Analyses

Continuous data were presented as median and interquartile range (IQR). Mann-Whitney *U* test was used to compare groups and the Kruskal-Wallis test was used for statistical comparison of > 2 groups. Categorical data were expressed as numbers and percentage and groups were compared using Pearson chi-square test or Fischer's exact test where appropriate. The level of significance was set at a p-value < 0.05. Analyses were performed using SPSS software version 22.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

End-ischemic MP Provides Better Protection of Hepatocellular Function

Portal vein and hepatic artery resistance during 2 hours of *ex situ* reperfusion were influenced by end-ischemic MP treatment. At the portal side (Figure 3A), the vascular resistance was significant lower after end-ischemic COR and SNP preservation in comparison to livers only preserved with SCS. Although the

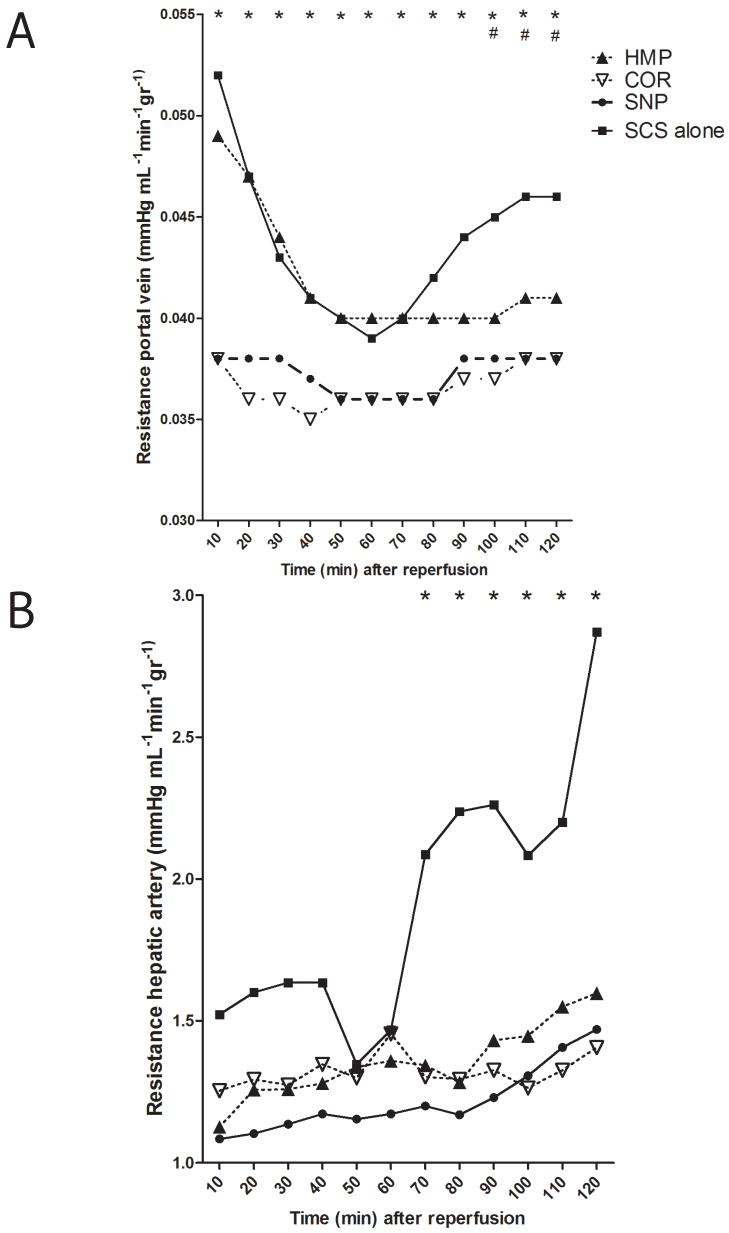

Figure 3: Vascular flow resistance in the portal vein and the hepatic artery during 2 hours of *ex situ* reperfusion. Panel A: During reperfusion, livers treated with end-ischemic MP controlled oxygenated rewarming (COR) and subnormothermic machine perfusion (SNP) had significantly lower portal vein resistance in comparison to liver preserved by SCS alone (*p<0.05). Livers treated with hypothermic machine perfusion (HMP) displayed a significantly lower portal vein resistance during the last 30 minutes of reperfusion, compared to SCS alone (#p<0.05). Panel B: During the last hour of reperfusion, livers that were preserved by SCS alone had a significant higher hepatic artery resistance compared to liver that underwent end-ischemic HMP, COR, or SNP (*p<0.05). Data are represented as medians.

portal vascular resistance after end-ischemic HMP was similar to the SCS alone group during the first hour of reperfusion, resistance during the second hour of reperfusion was significantly lower in the end-ischemic HMP group, compared to only SCS preservation. At the side of the hepatic artery, end-ischemic HMP, COR, and SNP resulted in a significant reduction of the vascular resistance during the second hour of reperfusion (Figure 3B). Taken the vascular resistance characteristics of the portal vein and hepatic artery during the second hour of reperfusion together, the results suggest better vascular hemodynamics after end-ischemic MP (HMP, COR, and SNP) compared to only SCS preservation.

After 2 hours of reperfusion, no statistically significant differences were found in the mitochondrial respiration and ATP production between the three end-ischemic MP groups. By adding ADP to stimulate ATP synthesis in the isolated mitochondria from livers that underwent one of the three temperature protocols of end-ischemic MP (HMP, COR, and SNP), the levels of oxygen consumption in mitochondria (state 3 respiration) were significantly higher compared to SCS alone (Figure 4A). In parallel, the RCR, a marker for mitochondria viability, was significantly higher in the three end-ischemic MP preserved groups (HMP, COR, and SNP), compared to preservation by only SCS (Figure 4B). In accordance with these isolated mitochondrial function analyses, cellular ATP levels were significantly higher in the three groups of end-ischemic MP compared to SCS alone (Figure 4C).

In line with the enhanced mitochondrial function after end-ischemic MP, also hepatic bile production after reperfusion was significantly higher in the three end-ischemic MP groups (HMP, COR, and SNP), compared to the group with SCS alone (Figure 5A). No significant differences in bile production were found between the three end-ischemic MP groups. Nevertheless, in all the end-ischemic MP groups' bile production during *ex situ* reperfusion remained lower than the *in vivo* bile production in the reference group. Biliary bilirubin concentrations were comparable among the three experimental groups (HMP, COR, and SNP), and *in vivo* reference group. However, the SCS preserved livers demonstrated significantly lower levels of biliary bilirubin (Figure 5B).

End-ischemic MP Reduces Hepatocellular Injury

Hepatocellular injury after *ex situ* reperfusion was significantly less in the three end-ischemic MP groups, compared to livers preserved by SCS alone. In the first 30 minutes of reperfusion, markers for hepatocellular injury, including levels of AST, ALT, and LDH in the perfusion fluid, were significantly lower in the end-ischemic MP groups (HMP, COR, and SNP), compared to livers preserved by SCS alone (Figure 6A). Similarly, after 2 hours of reperfusion, levels of the oxidative stress biomarker TBARS were significantly lower in the end-ischemic MP treated groups (HMP, COR, and SNP) (Figure 6B).

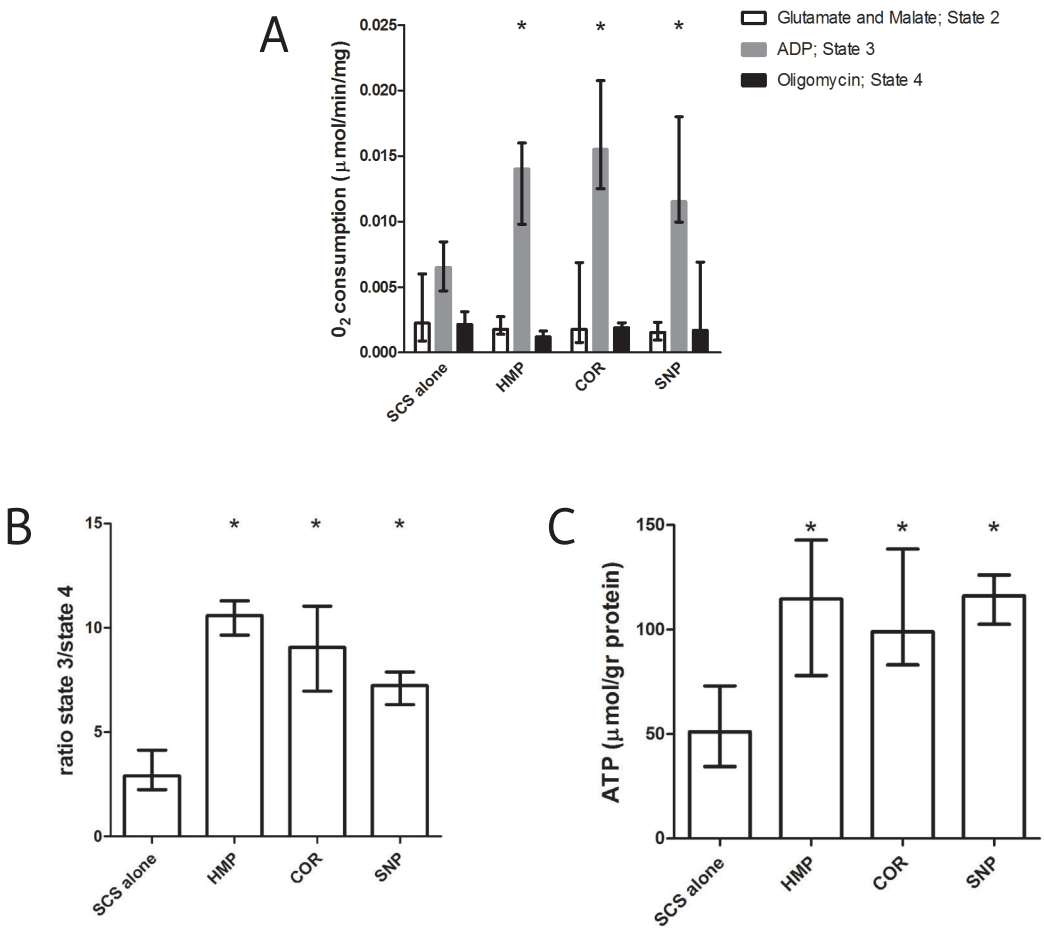

Figure 4: Mitochondrial respiration function and hepatocellular ATP concentrations after 2 hours *ex situ* reperfusion. Panel A: Oxygen consumption rates after the addition of adenosine diphosphate ADP (state 3 respiration) was significantly higher in livers that underwent 1 hour end-ischemic hypothermic MP (HMP), end-ischemic controlled oxygenated rewarming (COR) MP, and subnormothermic machine perfusion (SNP) compared to livers that underwent SCS alone (*p<0.05). There were no significant differences between the three end-ischemic MP groups. Panel B: The respiratory control ratio (RCR) was significantly higher in the three end-ischemic MP groups, compared to SCS alone (*p<0.05). The RCR value was not significantly different between the three end-ischemic MP groups. Panel C: Cellular ATP concentration (µmol/gr protein) after 2 hours of reperfusion was significantly higher in livers that first underwent 1 hour of end-ischemic MP in comparison to SCS alone (*p<0.05). Again, there were no significant differences in ATP production between the three groups with different temperatures protocols for end-ischemic MP (HMP, COR, and SNP). Data are represented as medians with IQR (error bars).

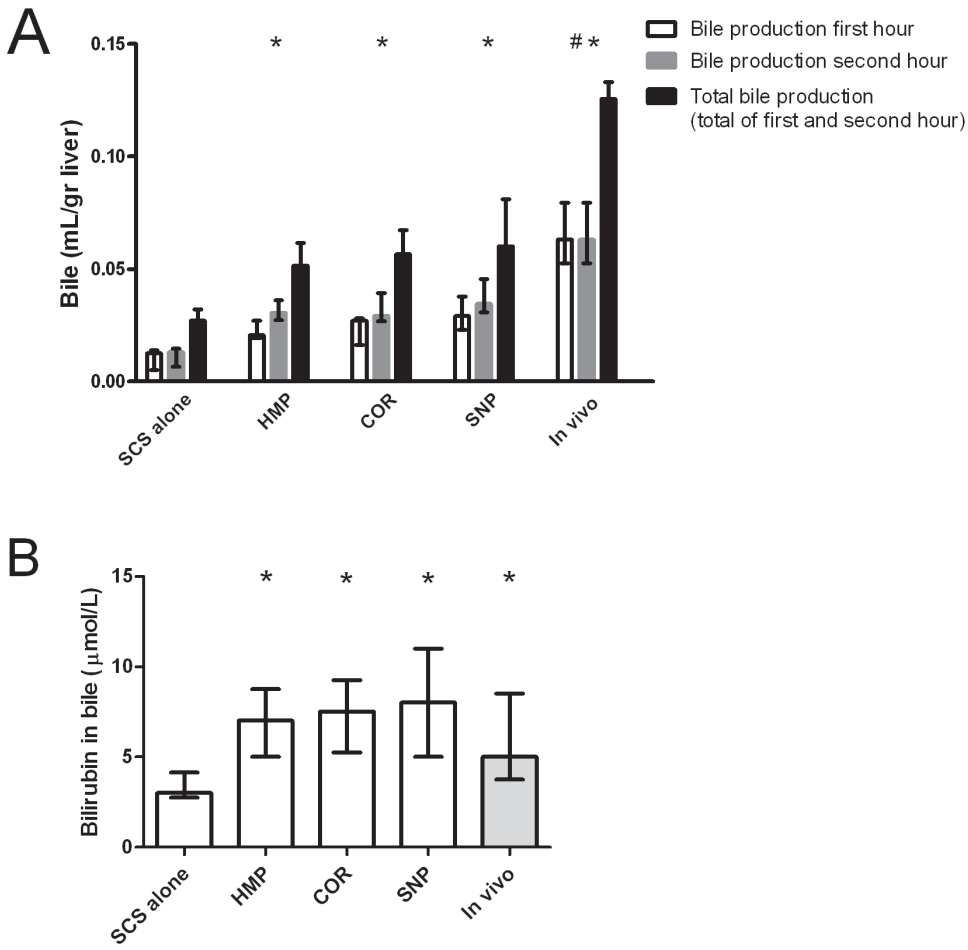

Figure 5: Bile production and bilirubin concentration in bile after 2 hours of *ex situ* reperfusion. Panel A: End-ischemic hypothermic (HMP), controlled oxygenated rewarming (COR) and subnormothermic machine perfusion (SNP) resulted in significantly higher bile volumes during the first and second hour of reperfusion compared to SCS alone (*p<0.05). There were no significant differences in bile production between the three end-ischemic MP groups. In general, however, bile production was significantly lower in livers that were preserved by HMP, COR, SNP or SCS alone, compared to values obtained *in vivo* (*p<0.05). Panel B: The concentration of biliary bilirubin was significantly lower in the group with only SCS preservation compared to the three MP groups and the reference group with *in vivo* bile measurements (*p<0.05). Between the three MP study groups and reference group no significant differences were measured in the biliary bilirubin concentration. Data are represented as medians with IQR (error bars).

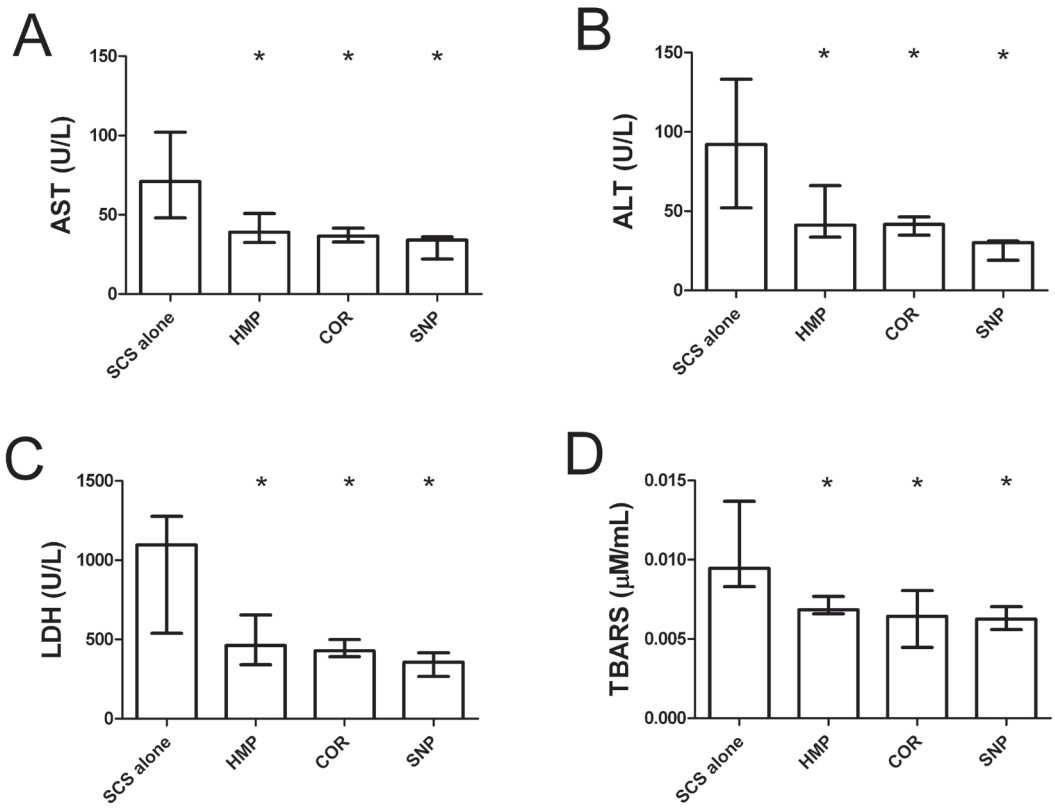

Figure 6: Biochemical markers of hepatocellular injury and a marker of oxidative stress (TBARS) measured in the perfusion fluid during 2 hours *ex situ* reperfusion. Panel A-C: Concentrations of AST, ALT, and LDH into the perfusion solution during the first 30 minutes of reperfusion. Concentrations of AST, ALT, and LDH during the first 30 minutes of reperfusion were significantly lower in livers that underwent end-ischemic hypothermic MP (HMP), controlled oxygenated rewarming (COR) MP, or subnormothermic MP (SNP), compared to livers that underwent only SCS (*p<0.05). There were no significant differences in levels of AST, ALT, and LDH between end-ischemic HMP, COR, and SNP preserved livers. Panel D: After 2 hours of reperfusion, levels of TBARS were significantly higher in the SCS alone preserved livers versus the end-ischemic MP treated groups (HMP, COR, and SNP) (*p<0.05). No differences in TBARS levels were noted between the end-ischemic MP groups. Data are represented as medians with IQR (error bars).

End-ischemic MP Improves Cholangiocyte Function and Reduces Cholangiocyte Injury

Biliary bicarbonate concentration after 2 hours of reperfusion was significantly higher in the three groups of end-ischemic MP (HMP, COR, and SNP), compared to the group with SCS alone (Figure 7A). No significant differences were observed

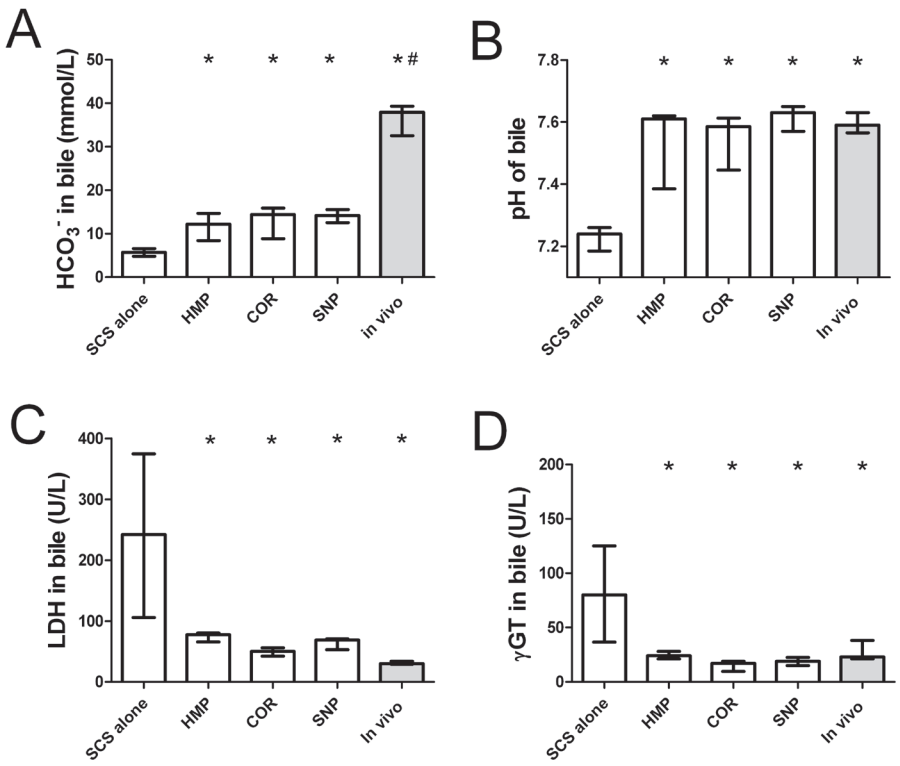

Figure 7: Biochemical markers of cholangiocyte function and injury measured in bile after 2 hours of *ex situ* reperfusion. Panel A and B: Markers of cholangiocyte function: bicarbonate (HCO_3^-) concentration and pH detected in bile. Biliary bicarbonate concentrations were significantly higher in livers that had been preserved by end-ischemic hypothermic MP (HMP), controlled oxygenated rewarming (COR) MP or subnormothermic MP (SNP), compared to livers that underwent only SCS (*p<0.05). There were no significant differences in biliary bicarbonate concentrations among the three machine perfusion groups (HMP, COR, and SNP). The concentration of biliary bicarbonate collected *in vivo* was significantly higher compared to the three end-ischemic MP groups (HMP, COR, and SNP) and the SCS alone group (*p<0.05). Panel B: The biliary pH was in the three end-ischemic MP groups comparable with the pH measured in the bile samples collected from the reference group with *in vivo* bile collection. Moreover, the livers with only SCS as preservation demonstrated significantly lower biliary pH than the three MP study groups and the group with *in vivo* bile collection (*p<0.05). There were no significant differences in biliary pH between the three end-ischemic MP groups. Panel C and D: The biomarkers for cholangiocyte injury, LDH and gamma-GT, were similar in the three end-ischemic MP groups (HMP, COR, and SNP) and the reference group with *in vivo* bile collection. In bile samples of livers preserved only with SCS, significantly higher values of LDH and gamma-GT were measured, compared to the end-ischemic MP groups and the group with *in vivo* collected bile (*p<0.05). Data are represented as medians with IQR (error bars).

between the three end-ischemic MP groups. However, in all four experimental groups (HMP, COR, SNP, and SCS alone) biliary bicarbonate concentrations after reperfusion were significantly lower compared to values obtained in bile samples from *in vivo* measurements (reference group). Biliary pH after 2 hours reperfusion was significantly higher in the three end-ischemic MP groups (HMP, COR, and SNP) versus the SCS alone group (Figure 7B). In fact, there was no significant difference in biliary pH between the three experimental MP groups and the *in vivo* reference group.

End-ischemic MP Provides Better Preservation of the Biliary Epithelial Lining of Large Bile Ducts

Injury of the epithelial lining of the lumen of large bile ducts was assessed by an established semi-quantitative histological grading using H&E staining. Livers that were preserved by SCS alone displayed significantly more grade 1 (< 50% of the circumferential lining) epithelial cell loss and significantly higher degrees of mural stroma necrosis (grade 1: < 25% and grade 2: 25-50%), compared with livers that underwent end-ischemic MP (HMP, COR, or SNP) (Figure 8 and 9). In fact, large bile duct biopsies of livers that underwent end-ischemic MP revealed only minimal injury of the epithelial cell layer and no signs of mural stroma necrosis. No differences were observed in the degree of vascular injury, incidence of vascular thrombosis, incidence of intramural bleeding, or degree of peribiliary gland injury among all study groups.

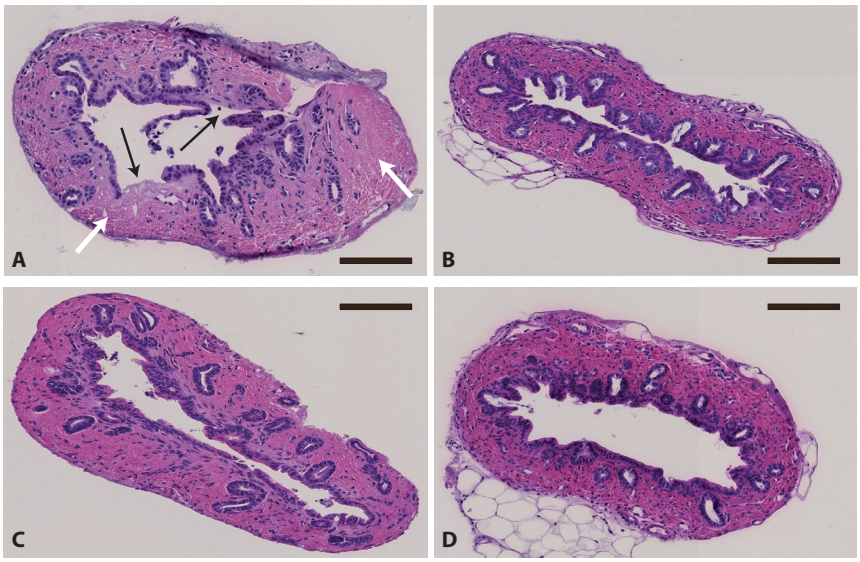

Figure 8: Hematoxylin and eosin (H&E) staining of the large bile duct biopsies after 2 hours *ex situ* reperfusion. Panel A: SCS preservation only. Black arrows indicate biliary epithelial cell loss (epithelium injury grade 1). White arrows indicate mural stroma necrosis (stroma necrosis grade 2). Panel B: End-ischemic hypothermic MP. Panel C: End-ischemic controlled oxygenated rewarming MP. Panel D: End-ischemic subnormothermic MP. Scale bars indicate 100 µm.

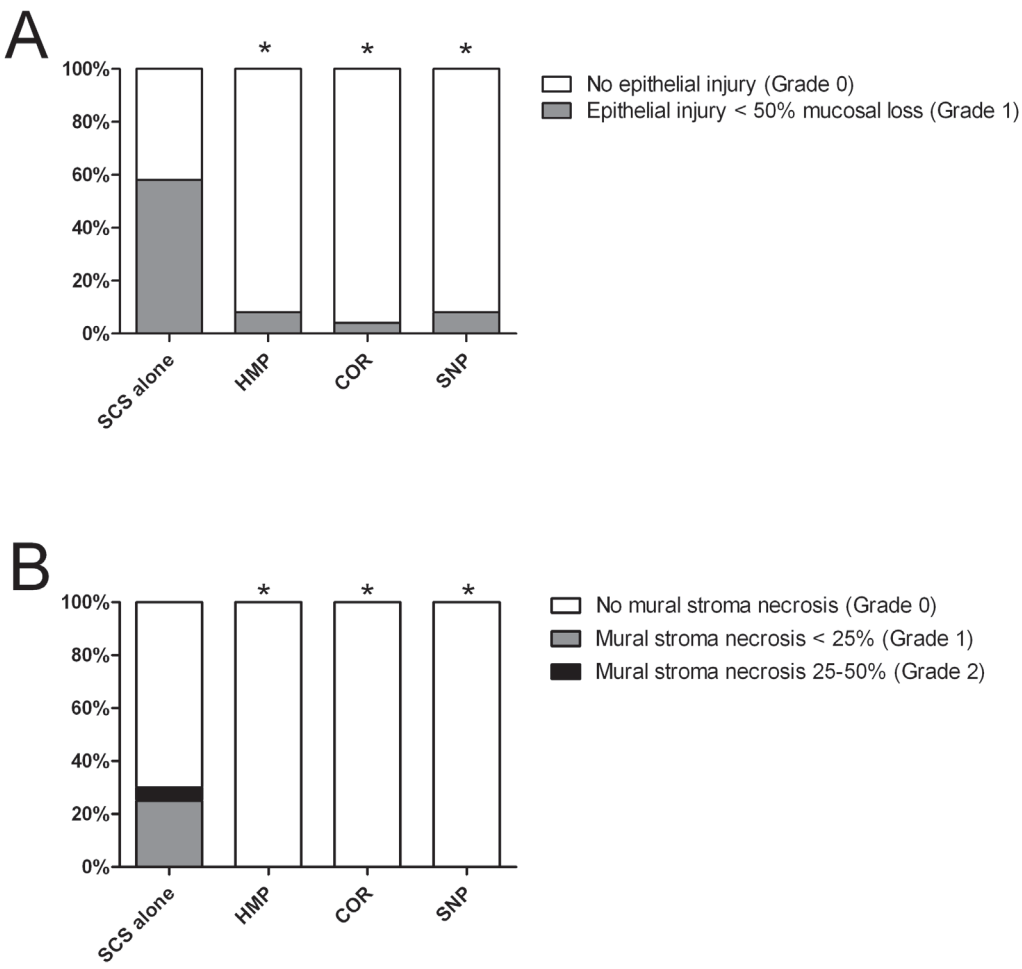

Figure 9: Overview of the distribution of epithelium injury and mural stroma necrosis of the large bile ducts. Panel A: Livers that were preserved by SCS displayed a significantly higher frequency of epithelium injury grade 1 (< 50% mucosal loss) (*p<0.05). Panel B: Livers that were preserved by SCS displayed significantly more mural stroma necrosis grade 1 (< 25% stroma necrosis) and mural stroma necrosis grade 2 (stroma necrosis 25-50%), compared with the three end-ischemic MP preserved groups (hypothermic MP, controlled oxygenated rewarming MP, and subnormothermic MP) (*p<0.05). There were no significant differences in epithelium injury or mural stroma necrosis between the end-ischemic MP groups.

Discussion

Currently, there are three different perfusion temperature conditions that are most frequently applied in the context of end-ischemic MP: hypothermic (<10°C), subnormothermic (20°C), and controlled oxygenated rewarming (8-20°C). However, these three methods have not been examined head to head, and it remains unknown which temperature protocol provides the best protection against biliary injury. In the current study, we, therefore, examined which perfusion temperature provides the best protection against bile duct injury in DCD donor livers.

Our study demonstrates that end-ischemic oxygenated MP significantly improves biliary function and morphology and reduces biliary cellular injury, compared to conventional SCS alone. Interestingly, this beneficial effect was independent of the temperature protocol used during end-ischemic MP. In addition, hepatocellular mitochondria oxygen consumption was enhanced after a short period of end-ischemic MP, resulting in higher cellular ATP concentrations. Moreover, end-ischemic MP mitigated hepatocellular injury during reperfusion and lowered oxidative stress, again independent of the temperature protocol used.

It is well known that biliary epithelial cells (cholangiocytes) and the bile duct stroma cells are very sensitive to ischemia and relatively short periods of ischemia result in a rapid depletion of intracellular concentrations of ATP (25,26). Due to ATP depletion, cholangiocytes lose their attachment to the basement membrane, resulting in sloughing of the epithelium layer and denudation of the bile duct luminal surface. In our study end-ischemic MP significantly minimized the degree of biliary epithelial cell loss and reduced the degree of mucosal stroma necrosis in the large bile ducts to a minimum, compared to only SCS preservation. Recently, three independent clinical studies have demonstrated that major mucosal cell loss (> 50% biliary epithelial injury) and mural stroma necrosis (< 50% necrotic cells) of the large bile duct is present in more than 80% of human donor livers (both DBD and DCD) at the end of SCS and subsequent reperfusion (4,24,27). Although biliary preservation injury is apparently almost universally present, only a minority of liver recipients develop NAS after transplantation. This observation has led to the hypothesis that insufficient proliferation and regeneration of cholangiocytes from the peribiliary glands are important factors in the pathogenesis of NAS (3,4). In the current study, we did not observe differences in injury of the peribiliary glands between the groups with end-ischemic MP and only SCS preservation. These findings are in contrast with our histological study in human donor livers demonstrating substantial injury of the more peripheral layers of the bile duct wall, including the peribiliary biliary glands, after SCS preservation (4). This study indicated that the severity of bile duct injury decreases from the most central, periluminal layers toward the periphery of the bile ducts (4). This is compatible with the fact that blood supply to the bile ducts enters from the

periphery, resulting in the lowest oxygen tension in central structures situated near the lumen. Peribiliary glands are situated in the peripheral layers of the bile duct wall. Apparently, rat liver bile ducts are more resistant to ischemic injury than human bile ducts. Therefore, the rat DCD liver model used in the current study did not cause detectable injury of peribiliary glands. Although we did not observe differences in injury of the peribiliary glands between the groups, end-ischemic MP significantly minimized the degree of biliary epithelial cell loss and reduced the degree of mucosal stroma necrosis to a minimum, compared to only SCS preservation. In parallel with these differences in histopathological assessment, we observed significantly lower biliary concentrations of biochemical markers of biliary epithelial injury, such as gamma-GT and LDH in bile. Moreover, in contrast to livers that were preserved by SCS alone, all three groups of end-ischemic MP revealed a normal alkalotic biliary pH and improved bicarbonate secretion after reperfusion. Biliary secretion of bicarbonate by biliary epithelial cells is considered an important protective mechanism against the cytotoxic effects of hydrophobic bile salts, which is known as the "bicarbonate umbrella" (20,28). Early recovery of the biliary function after SCS is clinically relevant since hydrophobic bile salts have been shown to play a role in worsening biliary injury and subsequent formation of NAS after transplantation (29,30). Taken together, these findings indicate that a short period of oxygenated MP after conventional SCS not only reduces the amount of biliary epithelial injury after reperfusion, but also contributes to an early recovery of biliary bicarbonate secretion, which helps cholangiocytes to protect themselves against the cytotoxic effects of hydrophobic bile salts.

So far, temperature conditions for end-ischemic MP have only been examined in animal studies, without special focus on biliary viability (15-17). Minor and coworkers (15) used a pig model to study liver graft viability during 4 hours of *ex vivo* reperfusion after living donation. During reperfusion, end-ischemic MP with COR was identified as the most protective temperature strategy. Interestingly, bile production, an important marker for liver function, was only significantly improved by end-ischemic COR and not by HMP or SNP. Moreover, bile production after end-ischemic MP at hypothermic or subnormothermic conditions was almost similar to the group with only SCS preservation. In this respect, our findings are different from those made by Minor *et al.* as we did not observe significant differences in bile production between livers that underwent end-ischemic HMP, SNP, or COR prior to reperfusion. A possible explanation for this could be a difference in organ quality, due to variations in the donation models. Minor and colleagues used livers obtained from living donation procedures, whereas we have used livers from DCD donors, who had suffered a period of 30 min warm ischemia prior to donor hepatectomy. The main difference between a living a donor and a DCD donor is the inevitable period of warm ischemia after cardiac arrest in the DCD donor. During this period of warm ischemia the liver has to switch from aerobic

to anaerobic conditions for ATP production. However, anaerobic production of ATP is by far not enough to fulfill the metabolic demand of the liver. This results in a severe ATP depletion prior to cold preservation. In contrast, during living donation, the liver is directly flushed with cold preservation solution, reducing metabolism and ATP consumption (31,32). Therefore, we hypothesize that DCD liver grafts used in our experiment have developed a more severe oxygen debt and sustained more hepatocellular injury at baseline, compared to liver grafts from a living donor. Because of this higher degree of cellular injury in DCD grafts, the effects of resuscitation by end-ischemic MP may be different and even more pronounced, compared to end-ischemic MP in living donor grafts. Our data indicate that a short period of oxygenated machine perfusion after conventional SCS of liver grafts obtained from DCD donors results in effective restoration of mitochondrial function and cellular ATP concentrations, which is independent of the temperature at which the liver is perfused.

Some limitations of this study should be mentioned: we did not examine the effects of end-ischemic MP at body temperature (normothermic, 37°C). Other studies have demonstrated that short periods of normothermic MP after SCS are not better than SCS preservation alone. In fact, a short period of normothermic MP after conventional SCS may not be any different from immediate implantation and reperfusion of a liver graft in the recipient. In both situations the whole cascade of I/R injury will be activated without a conditioning or protective effect of MP (5,33,34). Another limitation of our study is the *ex situ* reperfusion model. Although this model, which has also been used by other groups (9,12,14,15), allowed us to assess viability and injury of hepatocytes and cholangiocytes, we did not confirm our findings in a transplantation model. In particular, a longer follow-up period is required to assess biliary complications, which can only be achieved in a transplantation study.

In conclusion, this is the first study demonstrating that end-ischemic oxygenated MP of DCD livers provides better preservation of biliary epithelial function and morphology, independent of the temperature at which MP is performed. By significantly reducing biliary injury, especially in the large bile ducts, as well as restoring ATP levels in DCD liver grafts prior to transplantation, end-ischemic oxygenated MP could improve outcome after DCD liver transplantation.

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